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RESEARCH ARTICLE

Hallmarks of RNA silencing are found in the smut fungus *Ustilago hordei* but not in its close relative *Ustilago maydis*

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Abstract RNA interference (RNAi) acts through transcriptional and post-transcriptional gene silencing of homologous sequences. With the goal of using RNAi as a tool for studying gene function in the related basidiomycete cereal pathogens *Ustilago hordei* and *Ustilago maydis*, we developed a general purpose RNAi expression vector. Tandem, inverted fragments of the GUS gene were inserted into this vector flanking an intron and used to transform engineered GUS-expressing haploid cells. Down-regulation of the GUS gene and production of siRNAs were seen only in *U. hordei*, even though corresponding GUS double-stranded RNA was detected in both species. Similarly, when the endogenous *bW* mating-type gene was targeted by RNAi, mating was reduced only in *U. hordei*. Our work demonstrates the feasibility of using RNAi in *U. hordei* and provides experimental support for the observed lack of RNAi components in the *U. maydis* genome. We hypothesize that the sharply limited transposon complement in *U. maydis* is a biological consequence of this absence.

Introduction

RNA silencing or interference (RNAi) has had a significant impact on many different fields and has challenged the central dogma of molecular biology. Early studies in plants demonstrated that transgenes could silence, *in trans*, homologous endogenous genes (Napoli et al. 1990; van der Krol et al. 1990). Soon after, a similar phenomenon was seen in *Neurospora crassa* and was termed "quelling" (Romano and Macino 1992). However, not until 1998 was double-stranded (ds) RNA shown to be responsible for initiating the silencing process (Fire et al. 1998). Since that time, a role for RNA as cell regulator has come to light and intense investigation has identified many of the components and pathways involved. From these studies, RNAi-like phenomena have been implicated in transcriptional, post-transcriptional as well as meiotic gene silencing (Mette et al. 2000; Sijen et al. 2001; Shiu et al. 2006). RNA has been demonstrated to guide DNA methylation, histone modification, and induce the silencing of transgenes, viruses, and transposons (Volpe et al. 2002; Hall et al. 2003; Sijen and Plasterk 2003). Additionally, specialized RNAi pathways utilizing micro (mi) RNA have evolved in plants and animals that play important roles in their development (Bartel 2004).

Although specialized RNAi pathways have been reported, a common mechanism has emerged. In general, dsRNA, sometimes formed by RNA-dependent RNA polymerases (RdRPs) (Cogoni and Macino 1999; Dalmay et al. 2000; Smardon et al. 2000), is cut into small 21–26 nt fragments by an RNaseIII endonuclease called Dicer (Bernstein et al. 2001). Dicer fragments or short interfering (si) RNAs subsequently become associated with a multiprotein complex termed RISC (RNA-induced silencing complex) containing a protein from the Argonaute–Piwi family that plays a central role in RISC activity (Liu et al. 2004; Rand et al. 2004). The mRNA population is then scanned by RISC for regions complementary to siRNA resulting in mRNA cleavage or translational repression depending upon the organism and pathway. Repression of translation is often

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associated with animal miRNA and is thought to occur due to a lack of complementarity in the central part of the miRNA (Doench et al. 2003; Zeng et al. 2003). Under translational repression, messages are targeted to subcellular compartments called P-bodies where they are degraded or stored (Liu et al. 2005; Pillai et al. 2005). Under stressful conditions, messages have been observed to leave P-bodies and resume translation (Bhattacharyya et al. 2006). Messenger RNA targeted for cleavage, however, have complementarity with the central region of the siRNA and are thought to be cleaved immediately.

The ability of RNAi to work *in trans* has been exploited as a tool for manipulating gene expression (e.g., Boutros et al. 2004; Nakayashiki et al. 2005; Travella et al. 2006). RNAi has been especially valuable for organisms in which transformation and subsequent generation of gene deletions by homologous recombination is difficult. An added advantage is that, silencing of a particular gene creates a series of mutants exhibiting a range of phenotypes because interference or knock-down is rarely complete. This allows functions of essential genes to be discovered that otherwise would be lethal when deleted. RNAi mutagenesis has proven to be useful in various basidiomycete fungi (Liu et al. 2002; Namekawa et al. 2005) and should also be a valuable tool in the smut fungi.

Smuts belong to the order Ustilaginales and are pathogenic basidiomycete fungi responsible for significant agricultural losses worldwide. The genus *Ustilago* infects members of the Poaceae, which include cereal crops and forage grasses. Infection typically involves mating of haploid yeast-like cells on the host plant surface resulting in a dikaryotic infection hypha that penetrates the surface and grows between plant cells in a biotrophic manner. Although smuts execute a variety of infection strategies, diseased floral tissue is a common feature. Some smuts, such as *Ustilago maydis*, can induce large tumors on floral and vegetative tissues of their host plant (corn, *Zea mays*) which in later stages fill with masses of dark teliospores. *U. maydis* is perhaps the best-known smut and has become a model for this group of fungi (Martinez-Espinoza et al. 2002; Kahmann and Kamper 2004). As a result of the extensive studies with *U. maydis*, a vast number of molecular tools are available including the genome sequence (Kamper et al. 2006). We are interested in its close relative, *Ustilago hordei*, because this pathogen has a genetically defined gene-for-gene resistance relationship with barley (Hu et al. 2002, 2003; Linning et al. 2004) which is not observed between *U. maydis* and corn. In order to exploit the advantages of RNAi, we developed an RNAi vector suitable for expression of dsRNA in *U. hordei* and *U. maydis*. We demonstrate the feasibility of RNAi mutagenesis in *U. hordei* and the lack of the RNAi phenomenon in *U. maydis* by targeting both a transgene and an endogenous mating-type gene.

Materials and methods

Vector construction

With the goal of using PCR products for cloning into both fungal and plant expression vectors, we decided to mimic the pMCG161 plant RNAi vector from ChromDB (http://www.chromdb.org; NCBI accession no. AY572837). To start, a 262 bp intron from the *U. hordei bW2* gene (NCBI accession no. Z18531) was placed between the Hsp70 promoter and terminator in the *Ustilago* vector, pUBleX1 (Hu et al. 2007); this episomal vector provides resistance to bleomycin/phleomycin/zeomycin-related compounds. For *Ustilago* species we routinely use selection on 50 µg/ml zeomycin (Zeocin, Invitrogen) in Complete Medium (CM) (Holliday 1974). The intron was flanked by restriction sites Asc1 and Avr2 upstream, and Sgf1 and Spe1 downstream, yielding pUBleX1-RNAi (Fig. 1a; Table 1, primers 1 + 2). The PCR of target gene fragments using primers containing *Asc1* and *Spe1* sites in one primer and Avr2 and Sgf1 sites in the other primer, resulted in products that could be

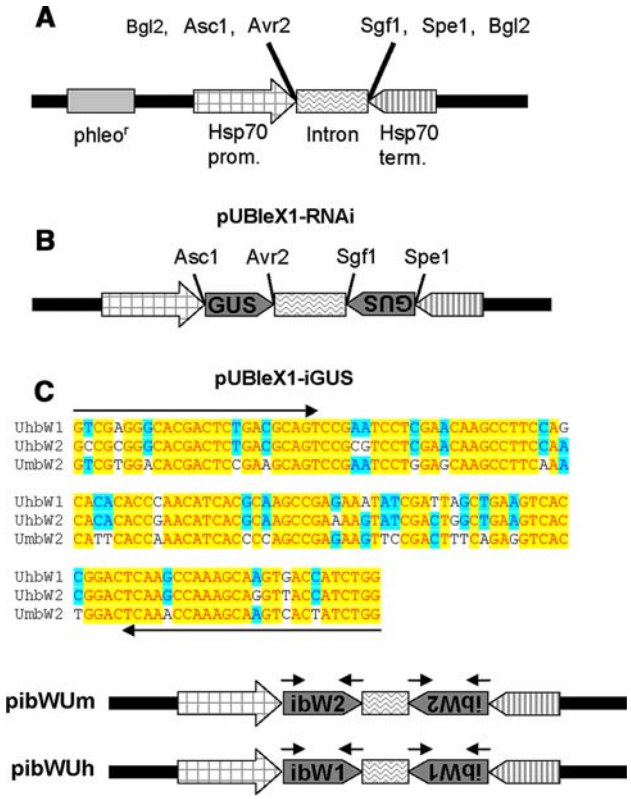

Fig. 1 Construction of RNAi vectors. **a** pUBleX1-RNAi plasmid. A 262 bp intron from the *U. hordei bW2* gene (accession no. Z18531) is flanked by cloning sites between the constitutive *Ustilago* Hsp70 promoter and terminator. **b** The pUBle-iGUS plasmid. A 563 bp GUS PCR product was ligated in inverted repeats to the pUBleX1-RNAi vector. **c** The pibWUm and pibWUh RNAi vectors. Homologous regions were obtained from respective *bW* alleles using PCR and ligated into pUBleX1-RNAi

Table 1 Primers for vector construction and quantitative RT-PCR

Primer	Sequence (5′–3′)	Description
1	ttAGATCTGGCGCGCCCCTAGGgtcagtaaagctttgtcctgtacgcgactc	Cloning; *U. hordei bW2* intron with Bgl2, Asc1 and Avr2 sites
2	ttAGATCTACTAGTGCGATCGCctgtagaaaggggatcagaacaagaaggg	Cloning; *U. hordei bW2* intron with Bgl2, Spe1 and Sgf1 sites
3	ttACTAGTGGCGCGCCtggtgatgtggagtattgcc	Cloning; 3′ end of GUS with Spe1 and Asc1 sites. nt 1,214[a]
4	ttGCGATCGCCCTAGGagttcatgccagtccagcgt	Cloning; 3′ end of GUS with Sgf1 and Avr2 sites. nt 1,777
5	ttACTAGTGGCGCGCCgtcgtggacacgactccgaagcagt	Cloning; *U. maydis bW2* with Spe1 and Asc1 sites
6	ttGCGATCGCCCTAGGccagatagtgacttgctttggtttga	Cloning; *U. maydis bW2* with Sgf1 and Avr2 sites
7	ttACTAGTGGCGCGCCgtcgagggcacgactctgacgcagt	Cloning; *U. hordei bW1* with Spe1 and Asc1 sites
8	ttGCGATCGCCCTAGGccagatggtcacttgctttggcttga	Cloning; *U. hordei bW1* with Sgf1 and Avr2 sites
9	gactttgcaagtggtgaatccgca	qPCR; for 5′ end of GUS. nt 696
10	ttcagcgtaagggtaatgcgaggt	qPCR; for 5′ end of GUS. nt 1,005
11	ggtgtcgacatgacagtctcgttgcg	qPCR; for *U. hordei* control gene. nt 1,415
12	ctcttcgaagtgttctgggagcagat	qPCR; for *U. hordei* control gene. nt 1,806
13	gtcgctattcaacgtcagcaacggtcttcg	qPCR; for *U. maydis* control gene. nt 3
14	gagcgaaaaggtgttctcgagcttctgtc	qPCR; for *U. maydis* control gene. nt 747

Restriction sites are in uppercase

[a] Gene location of 5′-most nucleotide (nt); see "Materials and methods" for GenBank accession numbers

ligated into either *Ustilago*-specific pUBleX1-RNAi or pMCG161 in tandem inverted orientation flanking the intron. A 563 bp region towards the 3′-end of the GUS gene (nucleotides 1,214–1,777) was cloned into pUBleX1-RNAi to form the pUBleX1–iGUS plasmid (Fig. 1b; Table 1, primers 3 + 4). A 131 bp conserved region of the *bW* gene was amplified from the *U. maydis bW2* gene (NCBI accession no. M84182; nucleotides 675–805; Table 1, primers 5 + 6) and the *U. hordei bW1* gene (NCBI accession no. Z18532; nucleotides 488–618; Table 1, primers 7 + 8) and cloned into pUBleX1-RNAi to form the plasmids pibWUh and pibWUm (Fig. 1c).

Strains and genetic transformation

U. hordei haploid strains Uh4857-4 (alias Uh364, *MAT-1*) and Uh 4857-5 (alias Uh365, *MAT-2*) (Linning et al. 2004) and *U. maydis* haploid strains 521 (*a1b1*) and 518 (*a2b2*) (Kronstad and Leong 1989) were used in this study. Stable GUS-expressing, transgenic lines were generated from *U. hordei* strain Uh4857-5 (*MAT-2*) and *U. maydis* strain 518 (*a2b2*) using a fungal expression vector containing a hygromycin selectable marker (Hu et al. 2002), and the bacterial *uidA* gene (*β*-glucuronidase or GUS). These GUS expressing lines were subsequently used for transformation with the pUBleX1–iGUS vector (Fig. 1b). Strains were cultured by shaking at 200 rpm in liquid CM at 22°C (*U. hordei*) or 28°C (*U. maydis*). For analysis of RNAi on the endogenous *bW* mating-type genes, plasmid pibWUh was transformed into *U. hordei* strain Uh4857-4 and plasmid pibWUm was transformed into *U. maydis* strain 518. RNAi lines generated from strain Uh4857-4 were tested for mating against *U. hordei* lines Uh4854-10 (*MAT-2*) and Uh4857-4 (*MAT-1*), while lines generated from *U. maydis* strain 518 were tested against strains 521 (*a1b1*) and 518 (*a2b2*). Mating was done by mixing 5 µl of each culture on 1.5% agar plates containing double complete medium (DCM) supplemented with 1.0% (w/v) activated charcoal (Holliday 1974). Biolistic transformation was done as described for *Cryptococcus neoformans* (Davidson et al. 2000). In brief, *U. hordei* haploid cells were grown overnight in 50 ml of CM to a cell density of $OD_{600} = 1$. Cells were then pelleted and resuspended in 10 ml of CM; 200 µl was then spread on a 1.0% agar plate containing DCM medium and 1 M sorbitol. Plates were dried for at least 4 h with opened lids in a laminar air-flow hood before particle bombardment. *Ustilago maydis* was transformed using polyethylene glycol (PEG) after enzymatic removal of cell walls as previously described (Brachmann et al. 2004).

GUS fluorometic MUG assay

Basidiospore cultures were grown to an approximate density of $OD_{600} = 1$. One milliliter was removed from each culture for the GUS histochemical assay (Jefferson et al. 1987, data not shown) while the remainder was used for RNA and protein extraction. For quantitative GUS assays, basidiospores were pelleted by centrifugation, frozen in liquid nitrogen, and ground to a fine powder. Proteins were extracted by mixing 100 mg of ground basidiospores with 1 ml of GUS extraction buffer (50 mM sodium phosphate buffer at pH 7.0, 10 mM EDTA, 10 mM *β*-mercaptoethanol, 0.1% Triton X-100, and 0.1% sodium lauryl sarcosine). After thorough mixing, protein extracts were

centrifuged twice for 10 min at 14,000 rpm and 4°C to remove cell debris. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad) by mixing 20 µl of basidiospore extract with 180 µl of diluted reaction mix before spectrophotometry. Samples were read along with BSA protein standards. Care was taken to ensure that extracts were within the linear range of the assay. GUS activity was measured by incubating equal protein concentrations in GUS extraction buffer containing 0.8 M MUG (4-methylumbelliferyl β-D-Glucuronide (Sigma) at 37°C. After equal incubation times, each sample was stopped by addition of two volumes of stop solution (0.2 M sodium carbonate). Fluorescence was measured in triplicate from each biological replicate along with MU (4-methylumbelliferone (Sigma) as the standard using a SpectraMAX GeminiEM microtiter-plate reader (Molecular Devices) with emission, excitation, and cut-off filters set at 460, 365, and 420 nm, respectively. For each sample, nmol MU per mg of protein per minute of incubation time was calculated and compared to the positive control GUS cell line.

RNA extraction and detection

Basidiospore cultures were pelleted by centrifugation before rapid freezing in liquid nitrogen. Frozen pellets were then ground to a fine powder and stored at −80°C until extraction of either RNA or protein. The RNA was extracted using a modified RNeasy protocol (Qiagen). Briefly, RNA was extracted from ground basidiospores using RLT buffer containing 1% β-mercaptoethanol. After passing through a QIAshredder, one volume of 80% ethanol was added to the supernatant prior to loading an RNeasy mini column. After a short spin, long RNA (>200 nt) remained bound to the RNeasy column while short RNA (<200 nt) passed through. Columns containing longer RNA were washed and eluted as per the RNeasy protocol. To obtain shorter RNA, 1.4 volumes of 100% ethanol were added to the supernatant before loading to a second RNeasy mini column and processing as per the RNeasy protocol. Longer RNA (5 µg) was separated on a MOPS/formaldehyde gel (2% formaldehyde) before capillary blotting to Hybond-N+ as recommended by the supplier (Amersham Biosciences/GE Healthcare). For detection of dsRNA, 5 µg of RNA was treated with RNase A/T1 (1:100 dilution of RNase A/T1 in RNase digestion buffer; mirVana miRNA Detection Kit (Ambion) for 60 min at 37°C, precipitated with ethanol and subjected to electrophoresis in a MOPS/formaldehyde gel before blotting to Hybond-N+. A GUS PCR probe (Table 1, primers 3 + 4) was labeled with [^{32}P]dCTP (PerkinElmer) using the Rediprime II Random Prime Labelling System (Amersham Biosciences/GE Healthcare) and hybridized to membranes using ULTRAhyb buffer (Ambion) at 42°C. After washing twice for 5 min with 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 0.1% SDS (sodium dodecyl sulphate) followed by washing twice for 15 min with 0.1X SSC, 0.1% SDS (all at 42°C), membranes were analyzed using the Cyclone Plus Storage Phosphor System (PerkinElmer). Short RNA (10 µg) was separated in a 15% denaturing PAGE gel (8 M urea/1X TBE buffer: 89 mM Tris–borate, 2 mM EDTA, pH 8.0) and electro-blotted onto Hybond-N+. Probe generation, hybridization and detection were the same as for longer RNA, but washing was less stringent and included a single 5 min wash with 2X SSC at 37°C followed by two 15 min washes with 2X SSC, 0.1% SDS at 42°C.

Quantitative RT-PCR

Two microgram of RNA, extracted using the RNeasy kit and quantified using a Nanodrop ND-1000 spectrophotometer, was treated with DNaseI (Invitrogen) prior to cDNA synthesis using SuperScript II reverse transcriptase and random primers (Invitrogen). The cDNA was diluted (8×) before quantification by Q-PCR using SYBR Green and an Mx4000 cycler (Stratagene). Equal amounts of cDNA generated from quantified RNA were amplified using AmpliTaq Gold (Perkin Elmer). For each cell line tested, data represent the average of three separate RNA preparations from biological replicates. For measurement of GUS mRNA, samples were normalized to a housekeeping gene (Succinate dehydrogenase, NCBI accession no. XM_751898, for *U. maydis* and a putative vacuolar protein sorting-associated protein, NCBI accession no. AM118080, for *U. hordei*) and compared to control cell lines. Amplification involved 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 60 s at 63°C, and 60 s at 72°C. Fluorescent data was collected at the end of the 63°C annealing step in every cycle. Melting curves were done at the end of each run to verify the reliability of the results.

Protein extraction and detection

Proteins were extracted from ground frozen cells with two volumes of protein extraction buffer (10 mM KCl, 5 mM $MgCl_2 \cdot 6H_2O$, 400 mM sucrose, 100 mM Tris–HCl, pH 8.1, 10% glycerine, 0.007% β-mercaptoethanol) and centrifuged at 14,000 rpm for 5 min at 4°C. Supernatants, 100 µl each, were then mixed with one volume of 2× protein loading buffer (100 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulphate, 20% glycerine, 0.2% bromo-phenol-blue, 200 mM dithiothreitol) and boiled for 5 min before SDS-PAGE (8% acrylamide, 1.5 M Tris–HCl, pH 8.8). A test gel was stained with coomassie blue so that each sample could be visually quantified. For subsequent gels, equal amounts of protein extracts were loaded for each sample. Proteins

were transferred onto Sequi-Blot PVDF membrane (Bio-Rad) and probed with a rabbit polyclonal antibody against GUS (Molecular Probes A-5790) followed by a goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio/Can). Immunoreactions were detected using the ECL Western blotting detection system (Amersham Biosciences/GE Healthcare).

Results

Ustilago RNAi vector construction and expression

An RNAi expression vector was developed as an initial step in studying the efficacy of using RNAi as a tool for reducing gene expression in *Ustilago* species. The pUBleX1 expression vector (Hu et al. 2007) was chosen based on its utility for obtaining high expression levels for inserted genes and was engineered to contain an intron with flanking restriction sites between the constitutive Ustilago-specific Hsp70 promoter and terminator elements (Fig. 1a). To test the RNAi vector, GUS expressing lines were first obtained for both *U. hordei* and *U. maydis* (see "Materials and methods"). Next, a 563 bp region near the 3′-end of the GUS gene was amplified by PCR and digested with Asc1 and Avr2 for sense cloning and Sgf1 and Spe1 for anti-sense cloning into the RNAi vector (Fig. 1b). The episomal iGUS vector, pUBleX1–iGUS, was then introduced into haploid, GUS-expressing cell lines from both species and transformants were maintained by zeomycin selection. For confirmation that the pUBleX1–iGUS vector was functioning properly, the first step was to look for dsRNA corresponding to the cloned GUS fragments, since RNA expressed from this vector is designed to fold back upon itself. To do this, RNA was extracted from multiple RNAi lines and from GUS expressing control lines from both species. Next, an RNase protection assay (RNase A/T1-treated RNA) was conducted to eliminate all single stranded RNA. The RNase A/T1-treated RNA was then transferred to nylon membrane by Northern blotting and probed with a PCR product corresponding to the GUS fragment in the pUBleX1–iGUS vector. A single band of the expected 563 nucleotides was clearly seen in all RNAi lines carrying the pUBleX1–iGUS vector demonstrating the presence of GUS dsRNA and thus proper expression of the RNAi construct and subsequent folding of the transcript (Fig. 2). Indeed, no GUS mRNA or dsRNA were detected in the GUS expressing control lines (Fig. 2, + lanes). To compare starting amounts of material, undigested RNA was separated by electrophoresis and stained with ethidium bromide for visualization of rRNA (Fig. 2). Furthermore, in samples without RNase A/T1 digestion, no molecules could be detected at 563 nt while the GUS hairpin RNA migrated close to the 1,000 nt

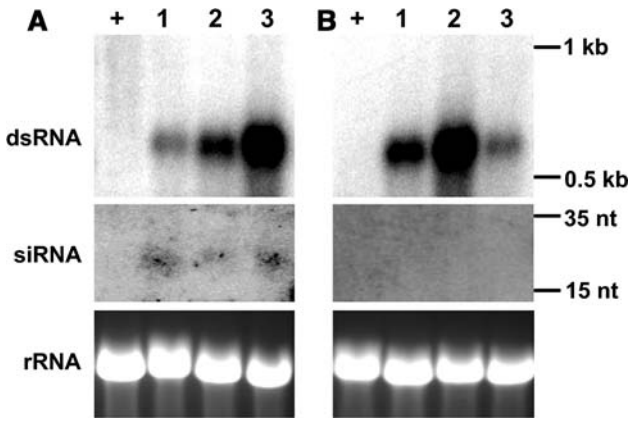

Fig. 2 Expression of dsRNA and production of siRNA. Transformants (1, 2, and 3) containing pUBleX1–iGUS were generated from stable GUS expressing (+) *U. hordei* (**a**) and *U. maydis* (**b**) lines. GUS dsRNA was detected after an RNase protection assay using RNase A/T1 in RNA only from the transformants (*upper panel*); this single product represents the double stranded hair-pin structure which migrates at the predicted length of 563 nt when denatured. siRNA in the 25 nt-range was detected only in *U. hordei* iGUS lines (*middle panel*). Equivalent amounts of RNA were used to start each experiment as shown by EtBr staining of rRNA (*lower panel*). The scale indicated on right is based on single-strand RNA markers (*upper panel*) and DNA oligonucleotides (*middle panel*): kb, kilobases; nt, nucleotides

marker, and in digested samples no more ethidium bromide-stained rRNA could be seen in the gel indicating successful RNase A/T1 digestion (data not shown). Since a distinguishing feature of RNAi is the generation of siRNA from dsRNA, the next step was to isolate and separate small RNAs for analysis. Northern blot analysis of PAGE-separated RNA probed with the same GUS fragment used to detect dsRNA, confirmed the presence of siRNA in *U. hordei* (Fig. 2). In contrast, after repeated efforts, no such GUS siRNA could be detected in *U. maydis* (Fig. 2).

RNAi is achieved through the RISC-guided cleavage of mRNA complementary to siRNA generated by Dicer (Liu et al. 2004; Meister and Tuschl 2004). To determine the influence of GUS dsRNA/siRNA on GUS mRNA levels, quantitative RT-PCR analysis was performed on cDNA from control and RNAi lines. Normalized data revealed a significant drop in GUS mRNA levels in *U. hordei* but not in *U. maydis* (Fig. 3a) indicating that siRNA in *U. hordei* effectively silenced the GUS transgene. The effects of RNAi could also be seen at the protein level as GUS enzymatic activity was significantly reduced in *U. hordei* RNAi lines as measured in a MUG fluorometric assay (Fig. 3b). In this assay, a decrease in GUS activity was measured as a marked reduction in enzymatic cleavage of MUG substrate in normalized protein extracts. The results of the MUG assay were supported by Western blot analysis of GUS protein (Fig. 3c). A GUS polyclonal antibody confirmed that GUS protein levels were concomitantly reduced in

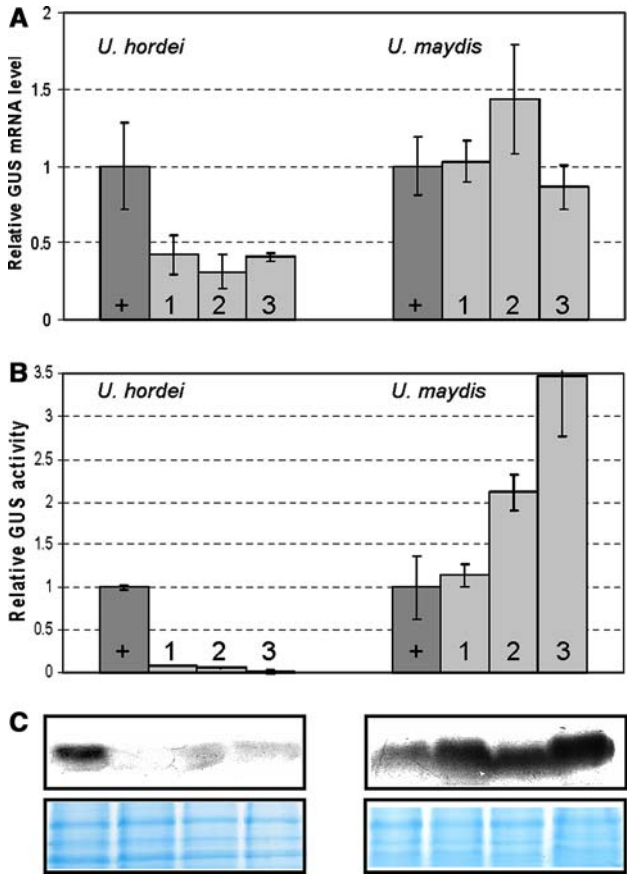

Fig. 3 RNA interference by dsRNA. **a** Presence of GUS dsRNA in transformants 1, 2, and 3 decreased the level of GUS mRNA in *U. hordei*, but not in *U. maydis* as measured by quantitative PCR. **b** MUG assay showing that GUS protein activity was significantly reduced in *U. hordei* iGUS lines but not in *U. maydis* iGUS lines. **c** Immuno blot showing that the GUS protein level was correspondingly reduced in *U. hordei* iGUS lines, but not in *U. maydis* iGUS lines (*upper panel*). Coomassie staining shows equal loading of protein (*lower panel*). +, stable GUS-expressing control lines

equivalent protein extracts from *U. hordei* RNAi lines (Fig. 3c), as indicated by a strong band of approximately 74 kDa only in the positive control GUS sample. Neither reduction in GUS protein activity nor level could be detected in *U. maydis* lines expressing the pUBleX1–GUS construct (Fig. 3b, c).

RNAi of an endogenous mating-type gene

Since RNAi proved to be successful in *U. hordei* for silencing the GUS transgene, our next goal was to measure its ability to silence an endogenous gene. Given that mating of compatible haploid strains can easily be visualized on charcoal-containing medium in the form of white mating hyphae, a domain of the *bW* mating-type genes, conserved among different alleles (Fig. 1c), was targeted in both *U. hordei* and *U. maydis*. Primers flanking a 131 bp region were designed for both species and PCR products were ligated into the episomal RNAi vector, pUBleX1-RNAi (Fig. 1c and Table 1, primers 5–8). Transformants were obtained for both *U. hordei* Uh4857-4 (*MAT-1*) and *U. maydis* 518 (*a2b2*) and tested for their mating ability. For this assay, haploid cultures were placed as drops on the charcoal-containing medium (Fig. 4). To the left on the agar surface a drop of the haploid line to be tested was spotted. An equivalent drop was co-spotted with the haploid culture of either mating type in the center row and to the right, respectively. Successful mating resulted in white fuzzy colonies, whereas haploid colonies containing the same mating type remained smooth and grey. Three independent transformants showed a considerable reduction in the production of mating hyphae for *U. hordei* (Fig. 4a). Because the transgenic line was *MAT-1*, normal mating would have resulted when mixed with a *MAT-2* strain, as seen for the control (Fig. 4a, arrow). Reduced mating in the *U. hordei* RNAi lines indicated successful functioning of the pibWUh construct. Mating in four *U. maydis* transformants seemed to be unaffected since mating of all lines was indistinguishable from the control (Fig. 4b).

Discussion

In this paper, we described the construction of a functional RNAi vector for *Ustilago hordei* and demonstrated the feasibility of using RNAi to knock-down gene expression in this smut fungus. For our experimental design, we chose to

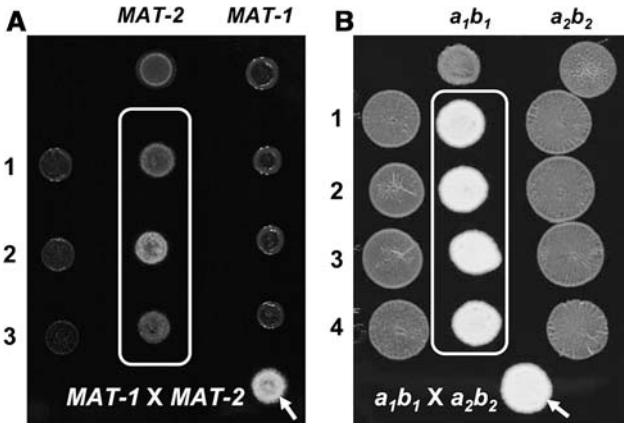

Fig. 4 RNA interference with the endogenous *bW* gene transcripts disrupts mating in *U. hordei* but not in *U. maydis*. Haploid transformants containing pibWUm or pibWUh were generated and tested for mating ability. **a** *U. hordei* ibWUh lines (1, 2, and 3, *MAT-1*) were paired against tester lines of mating type *MAT-1* and *MAT-2*. **b** *U. maydis* ibWUm lines (1, 2, 3, and 4, *a2b2*) were mated with tester lines *521* (*a1b1*) and 518 (*a2b2*). Successful mating produces dikaryotic aerial hyphae which appear as a white "fuzzy" colony phenotype on activated-charcoal plates. *White boxes*: cultures of compatible mating types; *arrows*: compatible mating controls. Shown are typical examples of test results which were repeated at least three times

separate sense and anti-sense PCR fragments by an intron since previous studies established an increase in RNAi efficiency with the use of an intron (Smith et al. 2000; Wesley et al. 2001); also for ease of transformation, we elected to express the hairpin constructs from a strong promoter on an episomal vector known to yield high expression levels (Hu et al. 2007). As anticipated, expression of dsRNA induced the RNAi pathway in *U. hordei.* This was evident by the down-regulation of a GUS transgene and presumably the endogenous *bW* mating-type genes. Occurrence of GUS-specific siRNAs provided experimental evidence for the presence of a Dicer gene in *U. hordei*. Additionally, down-regulation of transcripts homologous to the introduced dsRNA points to a functional RISC. Based on data from other basidiomycetes (Nakayashiki et al. 2006), this complex is likely to contain a protein in the Argonaute–Piwi family.

RNAi lines showed variation in the degree of silencing achieved, similar to reports on RNAi in other species (Tanguay et al. 2006; Walti et al. 2006). This was especially evident in the GUS histochemical assay where several silenced lines displayed a range of faint blue color indicating incomplete knock-down of the GUS transgene (data not shown). The quantitative MUG and RT-PCR results supported the histochemical assay and showed a reduction in steady-state mRNA levels of as much as 75% and a protein activity reduction to less than 1%. Such levels are consistent with those reported in other species (Liu et al. 2002). Incomplete knock-down of genes highlights the value of this technique as a tool for studying essential genes which might otherwise be lethal if deleted. When the endogenous *U. hordei bW1* mating-type gene was targeted by RNAi, a clear phenotype was seen upon mating in the form of a significant reduction in white mating hyphae. Even though knock-down was probably incomplete, a distinct phenotype was nevertheless seen. In addition, since each mating type possesses a *bE* and a *bW* allele which, upon mating, interact and produce an active bE1/bW2 and a bE2/bW1 heterodimer complex (Gillissen et al. 1992; Bakkeren and Kronstad 1993), it follows that the pibWUh construct must have affected both *bW* alleles in the dikaryon. Despite the 9.2% divergence between these sequences (12 out of 131 nucleotides, Fig. 1c), the RNAi construct seemed able to target both *bW* alleles. These results support the use of RNAi as a tool for targeting several members of a closely related gene family and demonstrate the feasibility of using RNAi to study gene function in *U. hordei.*

Previous studies have indicated that the RNAi phenomenon may be lacking in *U. maydis*. First, an earlier study showed that anti-sense transcripts fail to down-regulate the *pyr3* gene (Keon et al. 1999). More recently, components from the RNAi pathway could not be identified in the *U. maydis* genome (Kamper et al. 2006; Nakayashiki et al. 2006). However, since bioinformatic searches have failed in the past to identify RNA components for organisms known to posses RNAi, more direct experimental proof was needed. Our study is the first to demonstrate the presence of dsRNA upon introduction of the RNAi construct in *U. maydis* and to show the failure to detect corresponding siRNA. When the GUS gene was targeted by our RNAi vector, no negative effect was observed on GUS mRNA level, protein level, or gene function. Similarly, dsRNA targeted to the endogenous *bW* mating-type genes had no apparent negative effect on bW2 and bW1 functions, as normal mating ensued. These results support the previous *pyr3* report, as well as the bioinformatic studies, and suggest that no functional homolog to Dicer exists in *U. maydis*.

The results presented here are significant because *U. hordei* and *U. maydis* are phylogenetically closely related (Stoll et al. 2005). Comparison of DNA sequences between several large regions of the *U. hordei* genome and the corresponding regions of the *U. maydis* genome show conservation of synteny and a high level of sequence similarity (Bakkeren et al. 2006, unpublished). Furthermore, these two species have very similar life cycles and can be artificially forced to mate (Bakkeren and Kronstad 1996). Since a common ancestor to all eukaryotes is considered to have possessed the basic machinery for RNAi (Cerutti and Casas-Mollano 2006), one must ask why or how *U. maydis* lost its RNAi capability. Furthermore, what are the consequences of such a loss?

One immediate concern is the vulnerability to foreign or mobile genetic elements. First, how does *U. maydis* protect itself against attacks from viruses? RNAi is the prime safeguard used by plants against viruses, as evident in the fact that plant viruses contain suppressors of silencing to aid in their attack (Li and Ding 2001; Qu and Morris 2005). Furthermore, RNAi was recently shown to protect the chestnut blight fungus, *Cryphonectria parasitica*, from viral attack (Segers et al. 2007). Since viruses are known to infect *U. maydis* (Martinez-Espinoza et al. 2002), it remains to be determined what type of defence *U. maydis* employs and if such viruses have lost their suppressors of silencing. A second concern is the control of mobile genetic elements. Numerous studies in plants, animals, and fungi demonstrate the importance of RNAi-like mechanisms to control transposable elements (Nolan et al. 2005; Tran et al. 2005; Brennecke et al. 2007; Houwing et al. 2007). Surprisingly, the *U. maydis* genome is relatively devoid of transposable elements; in particular, no class II (DNA) elements or otherwise active endogenous elements can be found (Ladendorf et al. 2003; Kamper et al. 2006). On the other hand, *U. hordei* appears to contain a large number of transposable elements in its genome. Numerous transposable elements are evident throughout the genome as determined from sequencing 527 kb in the *MAT-1* region (Bakkeren et al.

2006) and 120 kb in a region containing an avirulence gene, *Avr1*, together with BAC end sequencing and genome hybridizations (Linning et al. 2004; Bakkeren, unpublished). This observation suggests that *U. maydis* has a different evolutionary history with transposable elements and may deploy a different but very effective strategy to control mobile genetic elements.

Another intriguing question is the possible consequence of RNAi loss on transcription regulation. It is tantalizing to think that transcription from both strands of a gene could occur coincidentally in a single cell without the expected degradation triggered by dsRNA. Recent reports on a number of organisms indicate that overlapping transcripts are more common than previously thought (Williams et al. 2005; Steigele et al. 2007). Such strategies may reflect more efficient utilization of genomic information and means of transcriptional control (Hongay et al. 2006). In support of these studies, *U. maydis* EST and SAGE libraries possess evidence of overlapping transcripts (Ho et al. 2007; J. Kronstad and G. Hu, personal communication).

Recent data for a number of diverse organisms point to life cycle stage-specific RNAi pathways (Nolan et al. 2005; Brennecke et al. 2007; Carmell et al. 2007; Gunawardane et al. 2007; Houwing et al. 2007). A recent report on *N. crassa* shows that the presence of dsRNA induces expression of RNAi components as well as numerous other genes including homologs of antiviral and interferon-stimulated genes, thus demonstrating an active response in vegetative cells (Choudhary et al. 2007). Additionally, *N. crassa* is known to silence unpaired DNA during meiosis, a process known as meiotic silencing of unpaired DNA (MSUD) (Shiu et al. 2001). Similarly, recent reports on mice and Drosophila suggest an ancient mechanism exists to silence unwanted DNA during meiosis (Brennecke et al. 2007; Carmell et al. 2007). Since we have shown that *U. maydis* does not possess a functional RNAi machinery, it remains to be seen how *U. maydis* deals with mobile genetic elements in vegetative cells and what *U. maydis* does with unpaired DNA during meiosis. Furthermore, it would be interesting to see which genes, if any, are up-regulated in the dsRNA iGUS lines in both *U. hordei* and *U. maydis.*

The loss of RNAi is not unique to *U. maydis*. Using bioinformatics on completed genomes, RNAi components could not be detected in the fungi *Saccharomyces cerevisiae* and *Candida lusitaniae* (Nakayashiki et al. 2006), in the parasites *Trypanosoma cruzi*, *Leishmania major*, *Plasmodium falciparum*, and in the red alga *Cyanidioschyzon merolae* (Cerutti and Casas-Mollano 2006). Intriguingly, the majority of these organisms, like *U. maydis*, are obligate parasites with relatively compact genomes and are devoid of the DNA class of transposable elements.

This study has shown that RNAi is a useful tool for the down-regulation of genes in *U. hordei*, and has raised a number of questions concerning an apparent lack of this phenomenon in *U. maydis*. Our laboratory is collaborating on a project to sequence the *U. hordei* genome. We anticipate identification of homologs to Dicer, Argonaute–Piwi, RdRP and perhaps MSUD genes from the genome sequence. Since a high degree of synteny has been observed between *U. hordei* and *U. maydis*, it will be interesting to compare *U. maydis* syntenic regions to *U. hordei* loci containing RNAi components. Such a comparison may provide clues to the evolutionary history of RNAi in *U. maydis*.

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